IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Stoynova et al.	
	Art Unit: [to be assigned]
Application No.: 10/654898	Examiner: [to be assigned]
Filing Date: September 5, 2003	Atty. Docket: US-100
Title: Method for Producing L-Amino Acid Using Bacterium Belonging to the Genus *Escherichia*, Lacking Active *mlc* Gene	

CLAIM FOR PRIORITY UNDER 35 U.S.C. § 119 IN UTILITY APPLICATION

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Priority under 35 U.S.C. § 119 is hereby claimed to the following priority document(s), filed in a foreign country within one (1) year prior to the filing of the above-referenced United States utility patent application (35 U.S.C. § 172):

Country	Priority Document Appl. No.	Filing Date
Russia	2002123822	September 6, 2002

A certified copy of the listed priority document and a translation of the front page is submitted herewith. Prompt acknowledgment of this claim and submission is respectfully requested.

Respectfully submitted,

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Date: January 6, 2004
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Reg.№ 20/12-400 July 23, 2003

CERTIFICATE

Federal Institute of Industrial Property (hereinafter referred as - Institute) do hereby certify, that attached materials are exact copy of the original specification, claims, abstract and drawings (if exist) of the application №2002123822 for issuing the patent for invention, filed to the Institute on September 6th, 2002 (06.09.2002)

Title of invention:	Method for producing L-amino acids using bacterium, belonging to the genus *Escherichia*, lacking active *mlc* gene.
Applicant:	Closed Joint Stock Company "Ajinomoto-Genetika Research Institute"
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РОССИЙСКОЕ АГЕНТСТВО
ПО ПАТЕНТАМ И ТОВАРНЫМ ЗНАКАМ
(РОСПАТЕНТ)

**ФЕДЕРАЛЬНЫЙ ИНСТИТУТ
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Наш № 20/12-400 «23» июля 2003 г.

СПРАВКА

Федеральный институт промышленной собственности (далее – Институт) настоящим удостоверяет, что приложенные материалы являются точным воспроизведением первоначального описания, формулы, реферата и чертежей (если имеются) заявки №2002123822 на выдачу патента на изобретение, поданной в Институт в сентябре месяце 06 дня 2002 года (06.09.2002).

Название изобретения: Способ получения L- аминокислот с использованием бактерий, принадлежащих к роду Escherichia, содержащих неактивный ген mlc.

Заявитель: Закрытое акционерное общество «Научно-исследовательский институт Аджиномото - Генетика»

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METHOD FOR PRODUCING L-AMINO ACID USING BACTERIUM, BELONGING TO THE GENUS ESCHERICHIA, LACKING ACTIVE *MLC* GENE.

Background of the Invention

Field of invention

The present invention relates to the microbiological industry, specifically to a method for producing L-amino acid using bacterium, belonging to the genus *Escherichia*, wherein *mlc* gene is inactivated.

Description of the related art

Mlc protein is a global regulator (repressor) of carbohydrate metabolism (Decker et al, Mol Microbiol 1998, 27:2:381-90; Kimata et al, Mol Microbiol, 1998, 29:6:1509-19; Plumbridge, Mol Microbiol, 1998, 27:2:369-80). It was shown Mlc protein regulates expression of several genes and operons. There are *ptsG* gene (Kimata et al, Mol Microbiol, 1998, 29:6:1509-19; Plumbridge, Mol Microbiol, 1998, 29:4:1053-63; Kim et al, J Biol Chem, 1999, 274:36:25398-402; Plumbridge, Mol Microbiol 1999, 33:2:260-73; Tanaka et al, Genes Cells, 1999, 4:7:391-9) encoding membrane-bound subunit, IICB(Glc), of glucose phosphotransferase system (PTS), ptsHIcrr operon (encoding general PTS proteins) Kimata et al, Mol Microbiol, 1998, 29:6:1509-19; Plumbridge, Mol Microbiol, 1998, 29:4:1053-63; Kim et al, J Biol Chem, 1999, 274:36:25398-402; Plumbridge, Mol Microbiol 1999, 33:2:260-73; Tanaka et al, Genes Cells, 1999, 4:7:391-9), manXYZ operon encoding enzyme II of mannose PTS (Plumbridge, Mol Microbiol, 1998, 29:4:1053-63), *malT* gene encoding the activator of maltose regulon (Decker et al, Mol Microbiol 1998, 27:2:381-90).

Genes of *mlc* regulon are also positively regulated by CRP-cAMP complex (Chapon and Colb, J. Bacteriol., 1983, 156:1135-43; Decker et al, Mol. Microbiol., 1998, 27:2:381-90; Kimata et al, Mol. Microbiol., 1998, 29:6:1509-19; Plumbridge, Mol. Microbiol., 1998, 27:2:369-80; Plumbridge, Mol. Microbiol., 1998, 29:4:1053-63; Kim et al, J. Biol. Chem, 1999,

274:36:25398-402; Plumbridge, Mol. Microbiol 1999, 33:2:260-73; Tanaka et al, Genes Cells, 1999, 4:7:391-9)

Regulation of *mlc* gene transcription is remarkably complicated. First, it is negatively regulated by Mlc protein itself. Unphosphorylated EIICB(Glc) (product of *ptsG* gene) can sequester Mlc protein from its binding site by direct protein-protein interaction and therefore induce expression of *mlc* regulon in response of glucose (Tanaka et al, EMBO J, 2000, 19:20, 5344-52; Lee et al, EMBO J, 2000, 19:20:5353-61; Nam et al, EMBO J, 2001, 20:3:491-8). Second, transcription of *mlc* gene is performed by two promoters P1 and P2 (Shin et al, J. Biol. Chem. 2001, 276:28:25871-75). Promoter P1 is recognized only by RNA polymerase, containing the housekeeping sigma factor σ^{70}($E\sigma^{70}$), while the promoter P2 can be recognized by both $E\sigma^{70}$ and $E\sigma^{32}$, containing the heat shock sigma factor. Thus *mlc* gene belongs to a class of genes, transcribed from the multiple promoters including one recognized by RNA polymerase associated with the alternative sigma factor in order to respond to various environmental conditions. In addition, a highly conserved CRP-binding site present within the *mlc* promoter (Shin et al, J. Biol. Chem. 2001, 276:28:25871-75).

Transport of carbohydrates provided by PTS may be the limiting step in overproduction of some amino acids. So, the inactivation of product of *mlc* gene, which negatively regulates PTS genes expression, seems to be necessary for increasing of amino acid production. In the populations of *Escherichia coli* growing in the glucose-limited environment the polygenic mutations in *mgl*, *mlc* and *malT* genes were found (Manch K., Genetics, 1999,153:1:5-12). But at the present time there is no report of inactivation of *mlc* gene used for producing of L-amino acid.

Disclosure of the Invention

An object of present invention is to enhance the productivity of L-amino acid producing strains and to provide a method for producing L-amino acid using these strains.

This aim was achieved by finding that the inactivation of *mlc* gene encoding the repressor of carbohydrate metabolism can enhance production of L-amino acid, such as L-threonine. Thus the present invention has been completed.

The present inventions are as follows:

1). An L-amino acid producing bacterium belonging to the genus *Escherichia*, wherein the bacterium has been modified to have *mlc* gene inactivated.

2). The L-amino acid producing bacterium, wherein L-amino acid is L-threonine.

3). A method for producing L-amino acid, which method comprises the steps of:

- cultivating the bacterium according to any 1 or 2 in a medium to produce and accumulate L-amino acid in the medium, and
- collecting L-amino acid from the medium.

4). The method according to 3), wherein L-amino acid is L-threonine.

5). The method according to 4), wherein the bacterium has been modified to have enhanced expression of L-threonine operon.

The present invention is described in details below.

1. Bacterium of the present invention

The bacterium of the present invention is an L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein the bacterium has been modified to have *mlc* gene inactivated.

In the present invention, "L-amino acid producing bacterium" means a bacterium, which has an ability to accumulate L-amino acid in a medium, when the bacterium of the present invention is cultured in the medium. The L-amino acid producing ability may be imparted or enhanced by breeding. The term "L-amino acid producing bacterium" used herein also means a bacterium, which is able to produce and accumulate L-amino acid in a culture medium in amount larger than a wild type or parental strain of *E. coli*, such as *E. coli* K-12 strain.

The term "a bacterium belonging to the genus *Escherichia*" means that the bacterium is classified as the genus *Escherichia* according to the classification known to a person skilled in the microbiology. As examples of the microorganism belonging to the genus *Escherichia* used in the present invention, *Escherichia coli* (*E. coli*) can be mentioned.

The bacterium belonging to the genus *Escherichia* that can be used in the present invention is not particularly limited, however for example, bacteria described by Neidhardt, F.C. et al. (*Escherichia coli* and *Salmonella typhimurium*, American Society for Microbiology, Washington D.C., 1208, Table 1) can be mentioned.

The term "*mlc* gene is inactivated" means that the target gene is modified in the way that the modified gene encodes a mutant protein with decreased capacity for regulation or such gene encodes completely inactive protein. It is also possible, that the modified DNA region is unable to provide the natural expression of Mlc protein due to deletion of a part of the gene or modification of adjacent region of the gene.

Inactivation of *mlc* gene coding for the repressor of PTS brings an increasing the supply of carbon source, such as glucose, into the cell of L-amino acid producing bacterium.

The *mlc* gene codes for Mlc protein (406 amino acid residues), which is a global repressor of carbohydrate metabolism. The *mlc* gene (gi:16129552; numbers 1665368 to 1666588 in the GenBank accession number NC_000913.1) is located between b1593 and *ynfL* genes on the chromosome of *E. coli* strain K-12.

Inactivation of the gene can be performed by conventional methods, such as mutagenesis treatment using UV irradiation or nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) treatment, site-directed mutagenesis, gene disruption using homologous recombination or/and insertion-deletion mutagenesis (Datsenko K.A. and Wanner B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12: 6640-45) also called as a "Red-driven integration".

L- threonine producing bacterium.

As a bacterium of the present invention, which has to be modified to have *mlc* gene inactivated, the L-threonine producing bacteria may be used.

The bacterium of the present invention may be improved by enhancing the expression of one or more genes involved in the L-threonine biosynthesis. Such genes are exemplified by genes of L-threonine operon, i.e. *thr* operon, which preferably comprises the mutant *thrA* gene, which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine; the *thrB* gene, which codes for homoserine kinase; the *thrC* gene which codes for threonine synthase. Another preferred embodiment of the bacterium is modified to enhance the *rhtA* gene, which codes for putative transmembrane protein. Another preferred embodiment of the bacterium is modified to enhance the *aspC* gene, which codes for aspartate aminotransferase (aspartate transaminase) (Russian patent application No. 2002104983). The most preferred embodiment of the bacterium is modified to increase expression amount of *aspC* gene, the mutant *thrA* gene, the *thrB* gene, the *thrC* gene, the *rhtA* gene and modified to inactivate *mlc* gene.

As a parent strain, the L-threonine producing bacteria belonging to the genus *Escherichia* such as *E. coli* strain VKPM B-3996 (US Patent 5, 175, 107, US patent 5,705,371), *E. coli* strain NRRL-21593 (US Patent 5,939,307), *E. coli* strain FERM BP-3756 (US patent 5,474,918), *E. coli* strains FERM BP-3519 and FERM BP-3520 (US patent 5,376,538), *E. coli* strain MG442 (Gusyatiner et al., Genetika (in Russian), 14, 947-956 (1978)), *E. coli* strains VL643 and VL2055 (EP 1149911 A) and the like may be used.

The bacterium of the present invention can be obtained by inactivation of in the bacterium inherently having ability to produce L- amino acid. Alternatively, the bacterium of present invention can be obtained by imparting ability to produce L- amino acid to the bacterium already having *mlc* gene inactivated.

Methods for preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of an oligonucleotide as a primer and the like may be ordinary methods well known to

one skilled in the art. These methods are described, for instance, in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989).

2. Method of the present invention

The method of the present invention is a method for producing L-amino acid, which method comprises the steps of cultivating the bacterium of the present invention in a culture medium to produce and accumulate L-amino acid in the medium, and collecting L-amino acid from the medium. Concretely, the method of the present invention is a method for producing L-threonine, which method comprises the steps of cultivating the bacterium of the present invention in a culture medium to produce and accumulate L-threonine in the medium, and collecting L-threonine from the medium.

In the present invention, the cultivation, the collection and purification of L-amino acid from the medium and the like may be performed in a manner similar to the conventional fermentation method wherein an amino acid is produced using a bacterium.

A medium used for culture may be either a synthetic medium or a natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the bacterium requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the used microorganism, alcohol including ethanol and glycerol may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate, and digested fermentative microorganism are used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like are used. As vitamins, thiamine, yeast extract and the like are used.

The cultivation is performed preferably under aerobic conditions such as a shaking culture, and stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 5-day cultivation leads to the accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then L-amino acid can be collected and purified by ion-exchange, concentration and crystallization methods.

Brief Description of Drawings

Figure 1 shows relative position of the primers mlcIL and mlcIR on plasmid pACYC184 used for amplification of *cat* gene.

Figure 2 shows the construction of chromosomal DNA fragment comprising inactivated *mlc* gene.

Best Mode for Carrying out the Invention

The present invention will be more concretely explained below with reference to Examples.

Example 1. Construction the strain with inactivated *mlc* gene.

1. Deletion of *mlc* gene.

Deletion of *mlc* gene was constructed by means of the method firstly developed by Datsenko and Wanner (Proc. Natl. Acad. Sci. USA, 2000, 97(12), 6640-6645) called as a "Red-driven integration". According to this procedure, the PCR primers mlcIL (SEQ ID NO: 1) and mlcIR (SEQ ID NO: 2) homologous to the both region adjacent to the *mlc* gene and gene conferring antibiotic resistance in the template plasmid were constructed. The plasmid pACYC184 (NBL Gene Sciences Ltd., UK) (GenBank/EMBL accession number X06403) was

used as a template in PCR reaction. Conditions for PCR were following: denaturation step for 3 min at 95 °C; profile for two first cycles: 1 min at 95 °C, 30 sec at 34 °C, 40 sec at 72 °C; profile for the last 30 cycles: 30 sec at 95 °C, 30 sec at 50 °C, 40 sec at 72 °C; final step: 5 min at 72 °C.

Obtained 935 bp PCR product (Fig. 1, SEQ ID NO: 3) was purified in agarose gel and used for electroporation of the *E. coli* strain MG1655, containing the plasmid pKD46 with temperature sensitive replication. The plasmid pKD46 (Datsenko and Wanner, Proc. Natl. Acad. Sci. USA, 2000, 97:12:6640-45) includes 2,154 nucleotides (31088-33241) DNA fragment of phage λ (GenBank accession No. J02459), containing genes of λ Red homologous recombination system (γ, β, exo genes) under control of arabinose-inducible P_{araB} promoter. The plasmid pKD46 is necessary for integration of the PCR product into chromosome of the strain MG1655.

Electrocompetent cells were prepared as follows: night culture of *E. coli* strain MG1655 grown at 30 °C in LB medium, supplemented with ampicillin (100 mg/l), was diluted in 100 times with 5 ml of SOB medium (Sambrook et al, "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)) with ampicillin and L-arabinose (1 mM). Obtained culture was grown with aeration at 30 °C to an OD_{600} of ≈0.6 and then made electrocompetent by concentrating 100-fold and washing three times with ice-cold deionized H_2O. Electroporation was performed using 70 μl of cells and ≈100 ng of PCR product. Cells after electroporation were incubated with 1 ml of SOC medium (Sambrook et al, "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)) at 37 °C for 2.5 h and after that plated onto L-agar and were growing at 37 °C to select Cm^R recombinants. Then to eliminate the pKD46 plasmid, 2 passages on L-agar with Cm at 42 °C were performed and the obtained colonies were tested for sensitivity to ampicillin.

2. Verification of *mlc* gene deletion by PCR.

The mutants, containing the deletion of *mlc* gene, marked with Cm resistance gene, were verified by PCR. Locus-specific primers mlcPL (SEQ ID NO: 4) and mlcPR (SEQ ID NO: 5)

were used in PCR for the verification. Conditions for PCR verification were following: denaturation step for 3 min at 94 °C; profile for the 30 cycles: 30 sec at 94 °C, 30 sec at 52 °C, 2 min at 72 °C; final step: 7 min at 72 °C. PCR product, obtained in the reaction with the cells of parental Mlc^+ strain MG1655 as a template, was 1492 nucleotides in length (Fig.2, SEQ ID NO: 6). PCR product, obtained in the reaction with the cells of mutant MG1655 Δmlc::cat strain as a template, was 1191 nucleotides in length (Fig.2, SEQ ID NO: 7).

3. Construction of threonine producing strain with inactivated *mlc* gene.

Threonine producing strain *E. coli* TDH7/pRT614 (VKPM B-5318, US patent 6,132,999) was transduced to Cm resistance by the standard procedure of P1 transduction (Sambrook et al, "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)). The strain MG1655 Δmlc::cat was used as a donor. The resulted strain TDH7Δmlc::cat/pRT614 was verified by PCR to have Δmlc::cat deletion by means of primers mlcPL (SEQ ID NO: 4) and mlcPR (SEQ ID NO: 5).

Example 2. Production of L-threonine by *E. coli* strain with inactivated *mlc* gene.

Both *E. coli* strain TDH7/pRT614 and TDH7Δmlc::cat/pRT614 was grown during 18-24 hours at 37 °C on L-agar plates containing streptomycin (50 μg/ml). Then one loop of the cells was transferred to 50 ml of L-broth of the following composition: trypton – 10g/l, yeast extract – 5 g/l, NaCl – 5 g/l. The cells (50 ml, OD_{540} – 0.12 o.u.) grown at 37 °C within 4 hours on shaker (140 rpm) was used for seeding 450 ml of the medium for fermentation. The batch fermentation was performed in laboratory fermenter having a capacity of 1.0 l under aeration (1/1 vvm) with stirring at a speed of 1200 rpm at 39 °C. The pH value was maintained automatically at 6.6 using 8% ammonia liquor. The results are presented in Table 1.

The composition of the fermentation medium (g/l):

Glucose	100.0
NH_4Cl	1.75

KH_2PO_4	1.0
$MgSO_4 \times 7H_2O$	0.8
$FeSO_4 \times 7H_2O$	0.01
$MnSO_4 \times 5H_2O$	0.01
Mameno(TN)	0.15
Betaine	1.0

Glucose and magnesium sulfate are sterilized separately. pH is adjusted to 6.6.

Table 1.

Strain	Amount of threonine, g/l	Yield, %	DCW, g/l	Cultivation time, h
TDH7/pRT614	12.6	13.3	16.6	22.8
TDH7Δmlc::cat/pRT614	16.9	18.2	16.8	21.7

As it is seen from Table 1, inactivation of the *mlc* gene improved the L-threonine accumulation by the L-threonine producing strain TDH7/pRT614.

SEQUENCE LISTING

<110> Ajinomoto-Genetika Research Institute

<120> METHOD FOR PRODUCING L-AMINO ACID USING BACTERIUM, BELONGING TO THE GENUS ESCHERICHIA, LACKING ACTIVE MLC GENE.

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: delta mlc::cat
fragment

<400> 7
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ccaaattgag atagcgcaaa ttttggtaga acagttaaaa aatgttaacc ctgcaacaga 180
cgaatcaaca aagaaccgtt atacatcgcg tctatacctg tgacggaaga tcacttcgca 240
gaataaataa atcctggtgt ccctgttgat accgggaagc cctgggccaa cttttggcga 300
aaatgagacg ttgatcggca cgtaagaggt tccaactttc accataatga aataagatca 360
ctaccgggcg tatttttga gttatcgaga ttttcaggag ctaaggaagc taaaatggag 420
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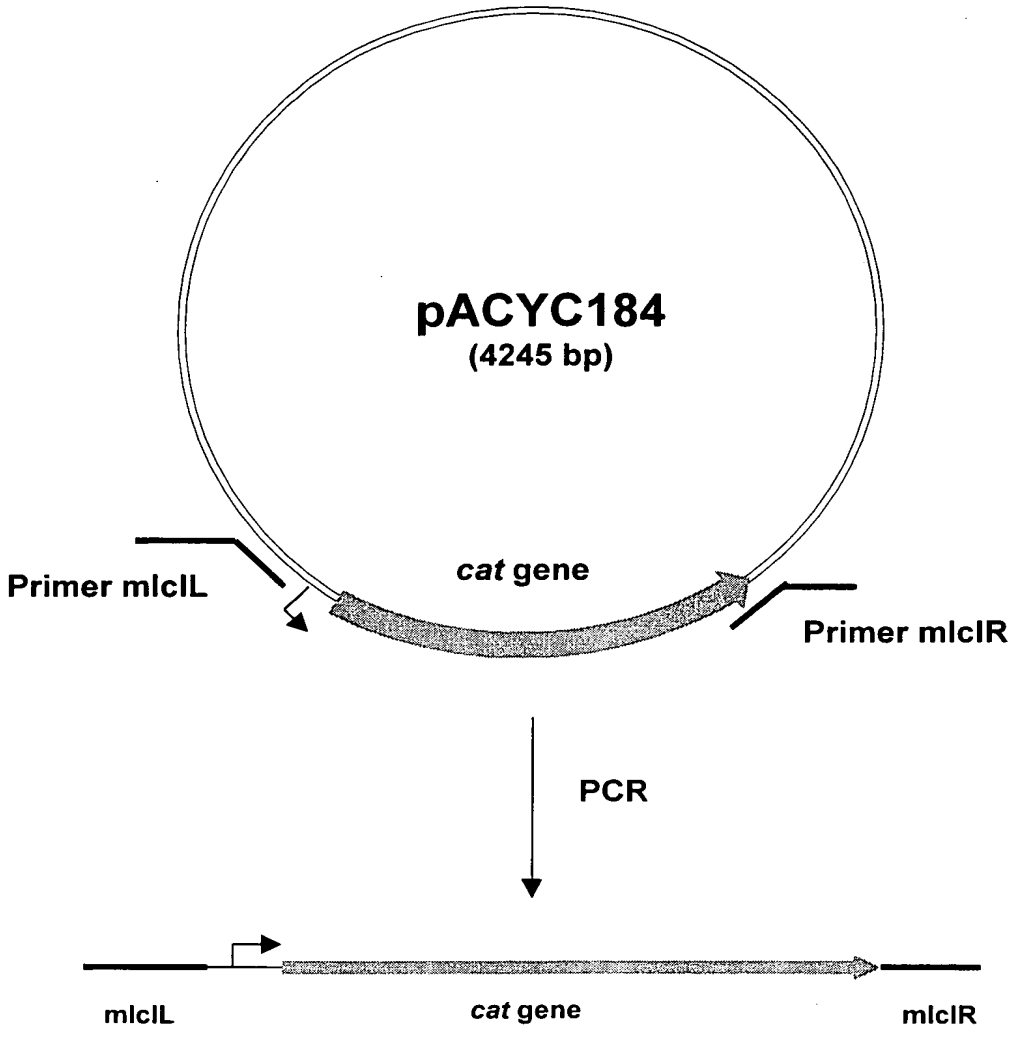
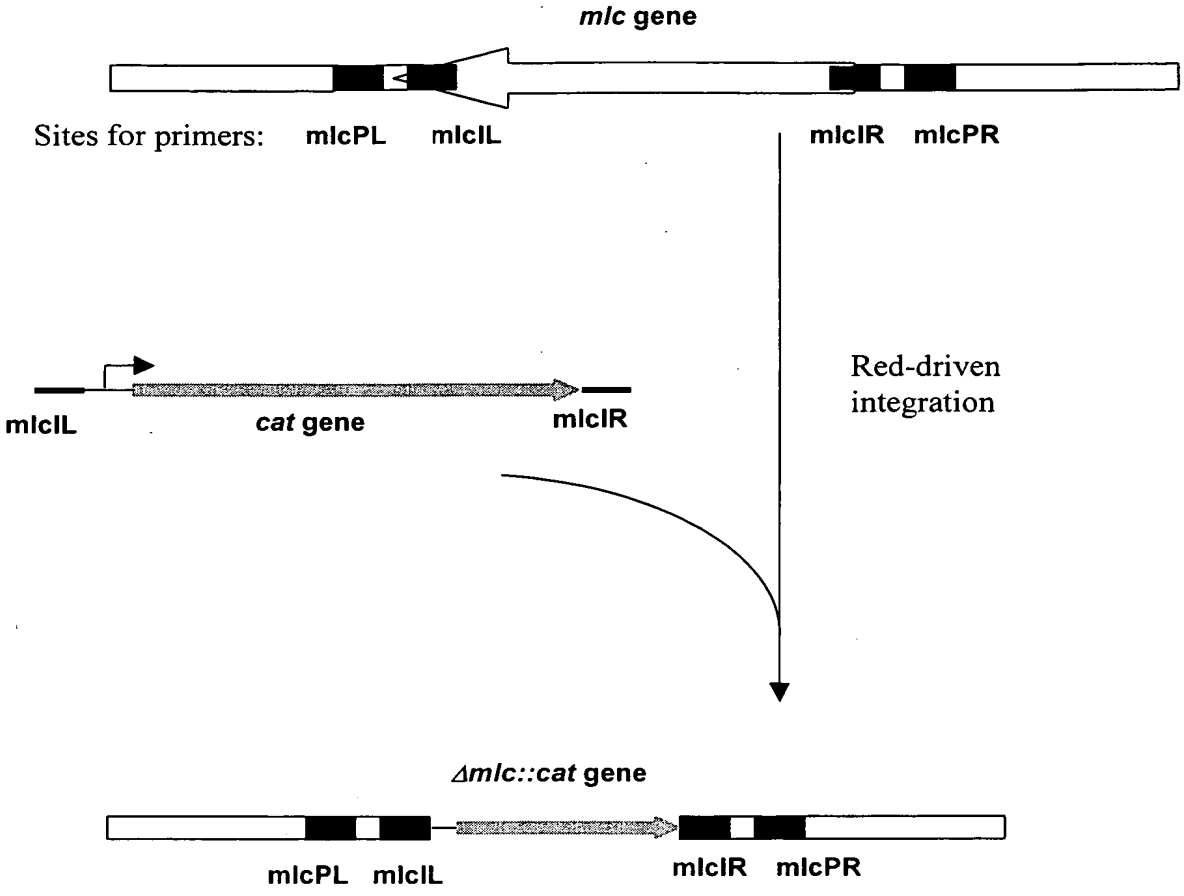
Fig. 1. Relative position of the primers mlcIL and mlcIR on plasmid pACYC184



Obtained PCR product (935 bp)

Fig. 2. Construction of chromosomal DNA fragment comprising inactivated *mlc* gene



What is claimed is:

1. An L-amino acid producing bacterium belonging to the genus *Escherichia*, wherein the bacterium has been modified to have *mlc* gene inactivated.
2. The L-amino acid producing bacterium, wherein L-amino acid is L-threonine.
3. A method for producing L-amino acid, which method comprises the steps of:
   - cultivating the bacterium according to claim 1 in a medium to produce and accumulate L-amino acid in the medium, and
   - collecting L-amino acid from the medium.
4. The method according to claim 3, wherein L-amino acid is L-threonine.
5. The method according to claim 4, wherein the bacterium has been modified to have enhanced expression of L-threonine operon.

Abstract of disclosure

There is provided a method for producing L-amino acid, such as L-threonine, using bacterium belonging the genus *Escherichia* with inactivated *mlc* gene coding for global regulator of carbohydrate metabolism.